Estrogen-Induced Protein. Time Course of Synthesis*

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ABSTRACT: A single injection of 17β-estradiol into immature rats or mature ovariectomized rats induces the increased incorporation of labeled amino acids into a specific soluble uterine protein (induced protein), detectable by starch gel electrophoresis.

The induced protein was not detected when uterine proteins were labeled with radioactive amino acids prior to estrogen. This indicates *de novo* synthesis of this protein and not changes in conformation or subcellular compartment. The induced protein is detectable after a lag period of 40 min following an injection of 17β-estradiol, when the synthesis of induced protein is measured under *in vivo* conditions. Induced protein continues to be synthesized at the high estradiol-induced rate for the period between 45 and 120 min after estradiol injection, whereas by 4 hr it appears to decline.

One of the early and critical effects of estrogen in promoting uterine growth is an increase in general protein synthesis in the uterus which occurs between 2 and 4 hr after estrogen administration (Noteboom and Gorski, 1963; Hamilton, 1964). In the first 2 hr after estrogen administration, general protein synthesis is not affected. However, increases in glucose metabolism, lipid, and RNA synthesis due to estrogen are all blocked by protein synthesis inhibitors at this early time (Noteboom and Gorski, 1963; Gorski and Axman, 1964). These observations led to the conclusion that early estrogen action involves synthesis of specific uterine proteins. Notides and Gorski (1966) have shown that estradiol administration to immature rats resulted in an early effect (30 min after intraperitoneal injection of estradiol) on the *in vitro* incorporation of labeled amino acids into one specific uterine-soluble protein identified by starch gel electrophoresis.

Certain questions about the increased labeling of this protein are the basis for this report. Kinetic studies of the estrogen-induced change in the rate of synthesis of the specific induced protein and a comparison of *in vivo* and *in vitro* labeling of uterine proteins are presented. Also, three possible effects of estrogen on the mechanism of the increased incorporation of labeled amino acids into specific protein are considered: (1) movement from an insoluble subcellular compartment, or (2) conformational changes in a uterine protein with a resultant electrophoretic mobility identical to the specific protein, or (3) induced synthesis of the specific protein.

Materials and Methods

Animals. Mature Holtzman (2.5–3 months) female rats were used 1–2 weeks after ovariectomy. When immature rats were used they were 22-days old. 17β-Estradiol (5 μg) in 0.5 ml of saline was injected intraperitoneally to immature rats and 10 μg in 1 ml of saline into mature rats; controls received saline alone. The animals were killed at various time intervals after estradiol injection, and their uteri were immediately cleared from surrounding fatty tissue and processed as indicated.

Double-Isotope Labeling of Uterine Proteins in Vitro. L-[^{3}H]Leucine (2.0 Ci/mmole, Schwartz) or L-[^{14}C]leucine (175 mCi/mmole, Schwartz) was used for labeling the uterine proteins. Immediately after killing, uteri were incubated in Eagle HeLa tissue culture medium at 37° for 2 hr under an atmosphere of 95% O_2–5% CO_2; estrogen-treated uteri were incubated with one isotope and the controls with the second. At the end of the incubation period, the uteri of both groups were combined, rinsed thoroughly with cold saline, homogenized in ice-cold 0.05% Na_2EDTA, centrifuged for 30 min at 15,000 rpm, and the supernatant was kept in a deep freeze until further examination.

Labeling of Uterine Proteins under in Vivo Conditions. Mature ovariectomized rats were anesthetized with ether, a small cut was made above the genital papilla, the lower portion of the uterus was elevated, and each horn was tied loosely with a thread just above the bifurcation of the uterus. Radioactive leucine (40 μl after neutralization of the acidic solvent with NaOH) was injected with a microsyringe into the lumen of the uterus (above the ligature), and the tie was immediately fastened to avoid leakage of the isotope (in cases where the uterine horn was not tied, most of the injected liquid leaked through the vaginal opening). At the end of the experiment, the animals were killed, and the uterine horn was cut open longitudinally and washed with 500 ml of ice-cold saline. The uteri were then homogenized in 0.05% EDTA and processed as described above. In some experiments, where incorporation into total uterine protein was determined, the uteri were homogenized in 5 ml of ice-cold 10% trichloroacetic acid, followed by centrifugation for 10 min at 2000 rpm. The precipitate was further washed three times with cold 5% trichloroacetic acid, ethanol, two times with ether, dried, and weighed. An aliquot of the 10% trichloroacetic acid supernatant was evaporated to dryness. The radioactivity in the acid-soluble and -insoluble material was determined after dissolving the proteins in 1 ml of NCS (Nuclear-Chicago).

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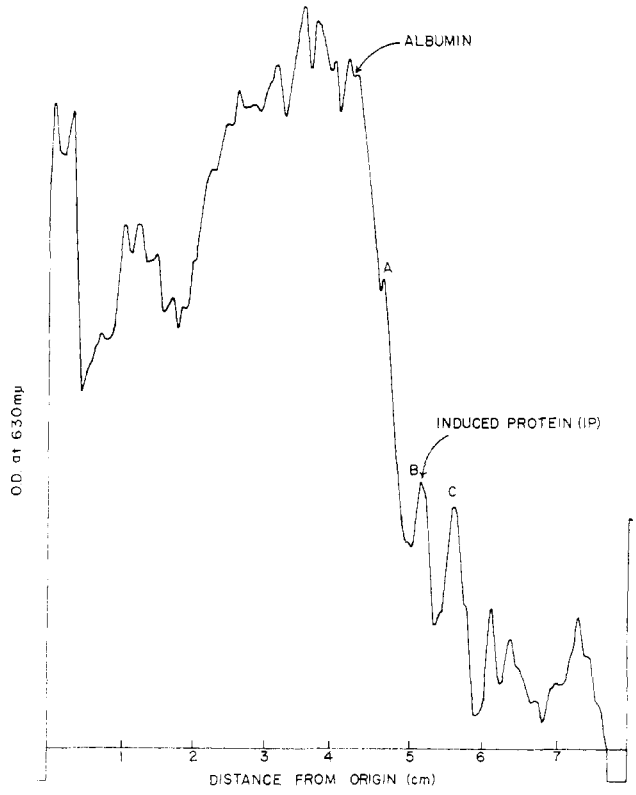

FIGURE 1: Densitometric tracing of uterine-soluble proteins separated by starch gel electrophoresis. Gel was stained with 1% Amido-Schwarz and scanned at 630 mμ.

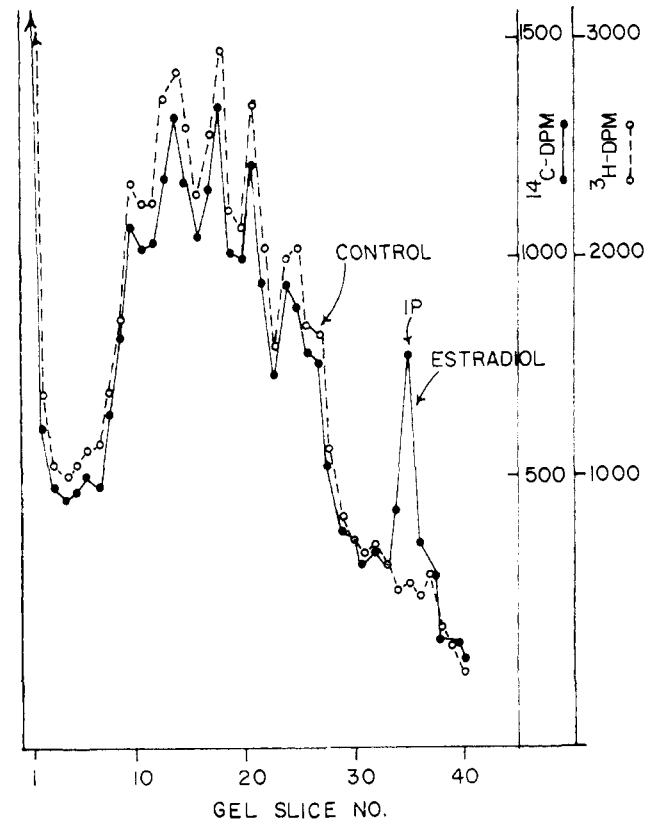

FIGURE 2: The electrophoretic distribution of soluble proteins from uteri incubated *in vitro* with labeled leucine. Five rat uteri, 30 min after injection of 5 μg of estradiol, were incubated with either [^{14}C]leucine (2 μCi; 175 mCi/mmole) or [^{3}H]leucine (5 μCi; 2.0 Ci/mmole) for 2 hr at 37°. The soluble proteins were separated by starch gel electrophoresis. (○----○) Controls with [^{3}H]leucine; (●——●) estradiol with [^{14}C]leucine.

Starch Gel Electrophoresis. Starch gels (14%) were made in TBE[1] at pH 8.6. After deaeration of the hot (about 90°) starch, the starch was sucked into glass tubes 0.8 × 12 cm, allowed to cool at room temperature, and placed in a cold room for 0.5 hr. The gels were ready for use 1 hr after preparation. To obtain the gel in a certain length, the gel was pushed out of each end of the glass tube and cut to the desired length (10 cm), thus obtaining a straight surface of the gel at both ends. The tubes were placed in the electrophoresis bath with their lower ends standing on several pieces of Whatman No. 1 filter paper to prevent the gels from sliding out. Samples (100–200 μl) were mixed with 25 μl of 15% Ficoll (Pharmacia) containing Canalco electrophoretic tracking dye. Electrophoresis was performed in TBE buffer at room temperature at 1.5 mA/tube for 3 hr. The gels were stained with 1% Amido-Schwarz in 7% acetic acid for at least 1 hr and then soaked in a large volume of 7% acetic acid overnight. The excess dye was removed with an electrophoretic decolorizer.

After destaining, the band corresponding to serum albumin was easily detected (Notides and Gorski, 1966). We observed three stainable bands migrating faster than the albumin when the gels were scanned with a Gilford spectrophotometer; the middle one (B) contained the induced protein (Figure 1). The migration distance from the top of the gel of albumin and each of the three bands was recorded for each sample.

[1] Abbreviation used is: TBE, 0.033 M Tris–0.01 M boric acid–0.0015 M Na_2EDTA.

Each gel was cut into 1.65-mm slices for counting. Each slice was placed directly in a counting vial, and 1 ml of NCS was added and incubated overnight at 45°. The slices were mixed vigorously with a Vortex mixer which breaks the gels into small pieces. Scintillator solution (10 ml) (0.5% 2,5-diphenyloxazole (Nuclear Equipment Chemical Co.) and 0.03% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] (Packard) in toluene) was added and the mixture was reincubated at 45° for another hour. By this time a clear solution was obtained. Radioactivity was counted in a Packard Tri-Carb scintillation spectrophotometer; the counting efficiency of ^{3}H was 28% and that of ^{14}C was 50%. The quenching was uniform from sample to sample as determined by external standards.

Results and Interpretation

Double-Isotope Labeling of Uterine-Soluble Proteins under in Vitro Conditions. The pattern of labeling of the uterine soluble proteins, after electrophoretic separation on starch gel, is presented in Figure 2. In this experiment, uteri from estradiol- (30 min) treated immature rats were incubated with [^{14}C]leucine (2 μCi; 175 mCi/mmole) and uteri from saline controls with [^{3}H]leucine (5 μCi; 2.0 Ci/mmole). Injection of estradiol resulted in an increase in the incorporation of [^{14}C]leucine into the band corresponding to the

induced protein compared with saline controls. Moreover, the ratio of $^{14}C:^{3}H$ in the gel slices equaled the ratio of $^{14}C:^{3}H$ in the incubation medium except that in those slices corresponding to induced protein the ratio of induced protein was three times as high (Figure 3). This indicates a specific effect of estradiol on the incorporation of [^{14}C]leucine only into the induced protein, while in the rest of the proteins on the gel, labeling is proportional to the concentration of the isotope in the incubation medium. Reversing the isotopes in the incubation media, *i.e.*, estradiol- (30 min) treated uteri incubated with [^{3}H]leucine and control with [^{14}C]leucine, gave similar results (Figure 3). This excludes the possibility of preferential uptake of one isotope by the induced protein. The induction of this specific protein was also observed when similar experiments were performed with adult ovariectomized rats.

Stimulation of Synthesis of Induced Protein by Estradiol. The results described above indicate an increase in the incorporation of label into the band corresponding to the induced protein. However, they do not answer the question whether the appearance of higher labeling in this particular band results from estradiol-induced synthesis of induced protein, or conformational changes in protein with a resultant electrophoretic mobility identical with that of induced protein, or possible movement to the soluble fraction from some other cellular compartment. The experiments designed to answer this question were performed in two steps: (1) Uterine proteins were prelabeled by intraluminal injection of [^{3}H]leucine (20 μCi/horn; 2.0 Ci/mmole) into uteri of mature ovariectomized rats. The incorporation of radioactivity into total tissue protein and acid-soluble fraction was measured over a period of 4–24 hr. The labeling of the acid-soluble fraction reached a very low stable level 8 hr after injection of [^{3}H]leucine. During the period of 4–24 hr after injection of isotope, no significant differences were found in the total counts of the acid-insoluble proteins nor in their specific activity. (2) The next experiment was conducted during the period of stabilized labeling of uterine proteins. In this experiment 17β-estradiol was injected into ovariectomized rats 17 hr after intraluminal injection of [^{3}H]leucine (20 μCi/horn; 40.0 Ci/mmole). Thirty minutes after estradiol injection, the uteri were transferred to incubation media containing [^{14}C]leucine (5 μCi; 175 mCi/mmole) for a period of 2 hr. The electrophoretic separation of the radioactive soluble proteins is shown in Figure 4. In the induced protein band of the estradiol-treated rats, a very dramatic peak of ^{14}C counts, originating from the *in vitro* incubation after estrogen, was observed compared with the very small one resulting from the preestradiol labeling with [^{3}H]leucine (Figure 4). The same results were observed in two other similar experiments. It is interesting that the patterns of labeling of the soluble proteins were very similar *in vivo* and *in vitro* except that the intraluminal injection of [^{3}H]leucine resulted in a higher degree of incorporation of label (^{3}H) into a protein (band C) migrating faster than induced protein compared with the ^{14}C counts in this band. This probably was due to some effect of the intraluminal injection, since the same peak was noted in the control as well as the estradiol-treated rats (Figure 4).

The fact that the induced protein was labeled only after the injection of estradiol, namely, during the *in vitro* incubation with [^{14}C]leucine, indicates that estradiol caused synthesis

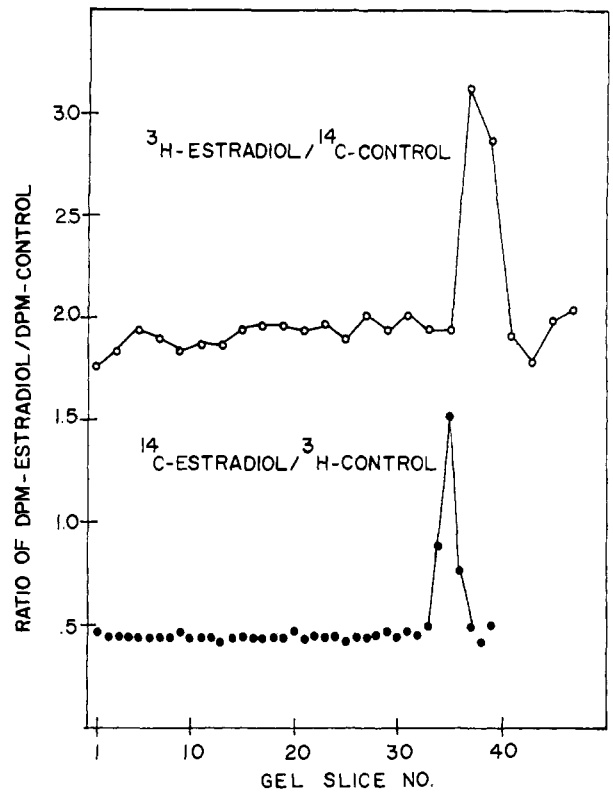

FIGURE 3: The ratio of radioactivity in soluble proteins from uteri of estrogen-treated *vs.* control rats. Experimental details as in Figure 2. Open circles represent ratio of ^{3}H (estradiol) to ^{14}C (control). Closed circles represent ratio from another experiment with ^{14}C (estradiol) *vs.* ^{3}H (control).

of the induced protein. Had the effect of estradiol been to cause conformational changes in another uterine protein, an increase in the preestradiol label of ^{3}H should have been observed in the induced protein band concomitant with the ^{14}C peak. The latter was not observed.

Time Course of Synthesis of Estradiol-Induced Protein. The time course of estrogen stimulation of induced protein synthesis in the past (Notides and Gorski, 1966) was done with *in vivo* estrogen treatment followed by 1- or 2-hr incubation with labeled amino acids. It was not possible to assess whether the estrogen was working both *in vivo* and *in vitro* and, therefore, the time course was not really known. In the following studies, *in vivo* labeling of uterine proteins, utilizing the intraluminal injection of labeled amino acids, was used to assess the rate of induced protein synthesis at various times after estrogen.

Figure 5 shows the incorporation of [^{3}H]leucine into the acid-soluble and -insoluble fractions during the first hour after intraluminal injection. The acid-soluble fraction was relatively highly labeled 15 min after intraluminal injection and declined rapidly to one-fourth by 60 min. The acid-insoluble proteins were labeled to a high degree by 15 min with incorporation of [^{3}H]leucine into proteins continuing to 30 min after intraluminal injection but leveling off at that time.

These data suggested that the induction of the synthesis of induced protein could be studied at very short time intervals after intraluminal injection of [^{3}H]leucine.

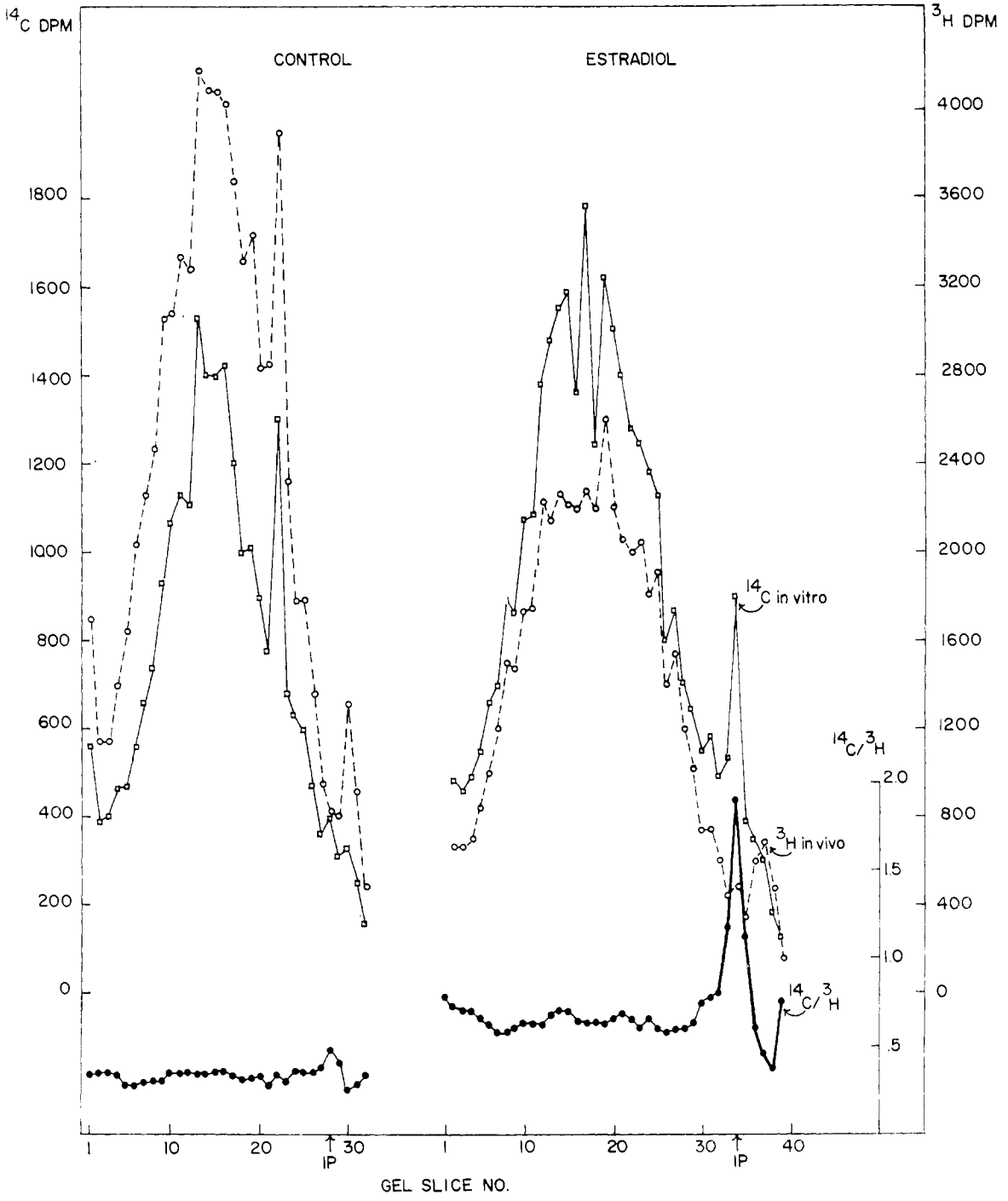

FIGURE 4: Electrophoretic distribution of uterine-soluble proteins. [^{3}H]Leucine (20 μCi/horn; 40.0 Ci/mmole) was injected into mature ovariectomized rats 17 hr before 17β-estradiol (10 μg). Thirty minutes after estradiol or saline injection the uteri were transferred to *in vitro* incubation medium containing [^{14}C]leucine (5 μCi/horn; 175 mCi/mmole) for 2 hr at 37°. (O----O) [^{3}H]Leucine *in vivo;* (□——□) [^{14}C]leucine *in vitro;* (●——●) ^{14}C:^{3}H.

In the following experiments, estradiol was injected into mature ovariectomized rats and the animals were killed at various time intervals (20–240 min) thereafter. Fifteen minutes before killing, each animal received an intraluminal injection of [^{3}H]leucine (20 μCi/horn; 40.0 Ci/mmole). The soluble proteins were separated on starch gel, and the rate of synthesis of induced protein was determined as the per cent of the area under induced protein peak of the total counts on the gel.

There is a lag of at least 25–40 min between the injection of estradiol and the earliest detectable increase in the synthesis of induced protein (Figure 6). The rate of induced protein synthesis peaks about 75 min after estradiol and continues to 2 hr. The rate appears lower at 4 hr, but by this time a

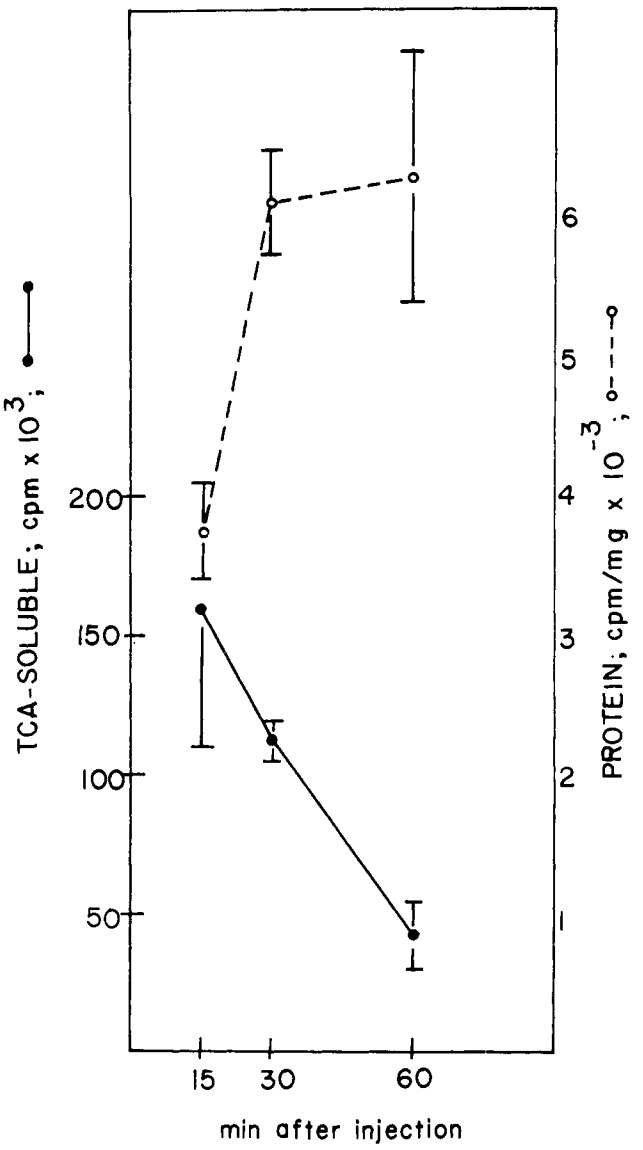

FIGURE 5: Incorporation of intraluminally injected [^{3}H]leucine into protein. [^{3}H]Leucine (3 μCi/horn; 40.0 Ci/mmole) was injected intraluminally into mature ovariectomized rats 15, 30, or 60 min prior to killing. Incorporation into total protein and the acid-soluble fraction is shown. Each point represents the average of six determinations with brackets representing standard error.

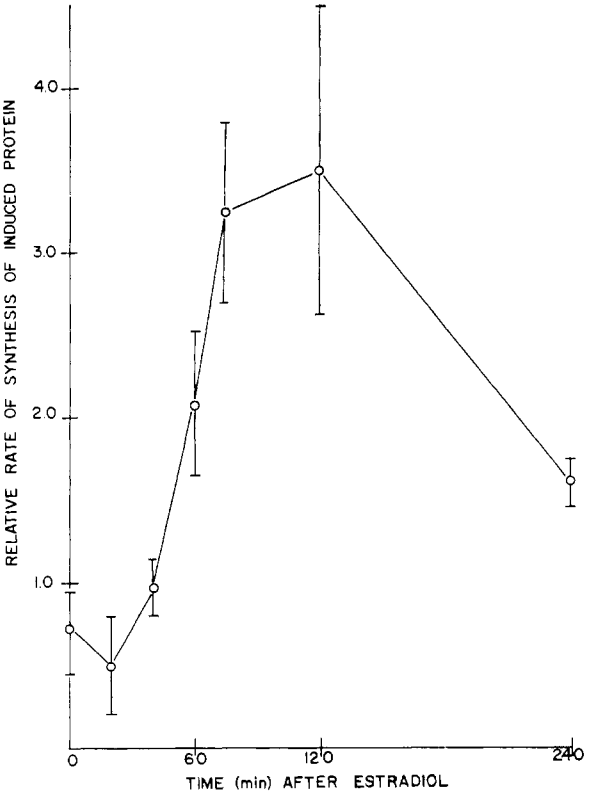

FIGURE 6: Time course of the induction of synthesis of the estradiol-induced protein. 17β-Estradiol (10 μg/rat) was injected into mature ovariectomized rats, and the animals were killed at various time intervals thereafter. Each animal received a single intraluminal injection of [^{3}H]leucine (20 μCi/horn; 40.0 Ci/mmole) 15 min before sacrifice. Each point represents the average of y-7 determinations ± standard error.

generalized increase in uptake of amino acids into protein occurs (Noteboom and Gorski, 1963).

General Discussion

The data described above present further evidence that estradiol stimulates *de novo* synthesis of a specific uterine protein, hence called induced protein. Earlier studies (Notides and Gorski, 1966) with cycloheximide, a protein synthesis inhibitor, had already suggested that estradiol induces the synthesis of this specific uterine protein. The technique used in this study, namely, the use of double-isotope labeling of the uterine proteins (preestradiol labeling with ^{3}H and postestradiol labeling with ^{14}C), confirmed this earlier assumption and excluded the possibility that the high degree of labeling of induced protein resulted from conformational changes caused by estradiol.

Other hormones have been shown to induce the synthesis of specific enzymes in mammalian tissues (Kenney *et al.*, 1968). The induction time of the synthesis of tyrosine transaminase in rat liver varies with respect to the inducer: 30–45 min after insulin or 1 hr after glucocorticoids. In the rat uterus, it was assumed (Notides and Gorski, 1966) that the effect of estradiol on the synthesis of induced protein was much faster, maximal by 30 min. However, in that study the investigators allowed a period of 1 hr after the initial 30-min postestradiol injection for the *in vitro* incorporation of isotope into the protein. The possibility existed that the effects of systemically administered estradiol persisted in the uterus while being incubated under *in vitro* conditions, leading to a false conclusion as to the time course of the effect of the hormone. That this possibility is a valid one is shown in the studies described in this paper. When the uterine proteins are labeled during a short time (15 min) under *in vivo* conditions, the injection of estradiol results in an initial lag phase of about 40 min followed by a marked increase in the rate of synthesis of the induced protein starting 45–60 min after estradiol administration. The induced protein continues to be synthesized at a high rate for at least 2 hr after estradiol injection; by 4 hr a lower rate of synthesis is noted, but at this time total protein synthesis increases

in the uterus and masks the response of induced protein (Noteboom and Gorski, 1963). The lag phase of about 40 min probably corresponds to the time necessary for the synthesis, transport to cytoplasm, and translation of the specific mRNA for the induced protein; since actinomycin D will block the synthesis of induced protein only if injected before estradiol. Actinomycin D injected 30 min after estradiol was no longer effective in blocking the synthesis of induced protein, suggesting a lag due to some posttranscriptional event (DeAngelo and Gorski, 1970).

These data support previous conclusions (Noteboom and Gorski, 1963), based on the use of protein synthesis inhibitors, that estrogen brings about specific protein synthesis which in turn brings about the numerous changes in uterine metabolism due to estrogen. Whether one or more of such induced proteins are involved is not known. The early glucose response to estrogen is also cycloheximide sensitive (Smith and Gorski, 1968); therefore, it would appear likely that increased rates of synthesis of other uterine proteins will be found. However, the synthesis of induced protein presently represents the only available system for such study.

In summary, a single administration of 17β-estradiol to immature rats or mature ovariectomized rats induces *de novo* synthesis of a specific uterine-soluble protein. This effect of estradiol is detectable under *in vivo* conditions within 45–60 min after injection of estradiol.

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Initiation of ϕX-174 RF-Primed Protein with *N*-Formylmethionine*

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ABSTRACT: Using the replicative form of deoxyribonucleic acid from the bacteriophage ϕX-174 to prime a cell-free system, we coupled transcription to translation and synthesized phage-specific protein. The coupled system incorporated formate from the formyl donor, N^{10}-formyltetrahydrofolic acid.

When the *in vitro* protein product was analyzed by polyacrylamide gel electrophoresis using ϕX-174 wild-type *in vivo* protein as an internal marker, its components comigrated with some of the phage specific proteins. All of the *in vitro* proteins incorporated formate. High-voltage electrophoresis of pronase-digested *in vitro* protein demonstrated that the formate was incorporated into *N*-formylmethionine.

Phage-specific protein can be synthesized in a cell-free system which couples transcription to translation (Bryan *et al.*, 1969). RF[1] from the bacteriophage ϕX-174 primes the "coupled system" which uses DNA-dependent RNA polymerase, ribosomes, and a soluble fraction prepared from uninfected *Escherichia coli*. During concomitant RNA and protein synthesis the entire genome is transcribed. The principal product, which is precipitable with anti-ϕX-174, seems to be a phage structural protein with a molecular weight of about 20,000 (Gelfand and Hayashi, 1969a).

This system provides an excellent opportunity to study the initiation of protein synthesis. The experiments which showed that fMet is an initiator of protein synthesis (Adams and Capecchi, 1966; Capecchi, 1966; Clark and Marker, 1966; Viñuela *et al.*, 1967; Webster *et al.*, 1966) used only RNA as the template, either RNA from RNA bacteriophages or endogenous *E. coli* mRNA. We now extend these observations and show that the initiation codons of a DNA phage are correctly transcribed and translated *in vitro* resulting in the incorporation of fMet into genome-specific protein.

* From the Department of Biology, University of California, San Diego, La Jolla, California 92037. *Received November 4, 1969.* R. N. B. is a United Cerebral Palsy Research and Education Foundation Postdoctoral Fellow. M. H. is a recipient of a Research Career Development award from the U. S. Public Health Service (GM-42360). This paper is number III in a series entitled: DNA-Dependent RNA-Directed Protein Synthesis *in Vitro*. This work is supported by U. S. Public Health Service Grant GM-12934 and National Science Foundation Grant GB-11783.

[1] The following abbreviations are used: RF, replicative form DNA; EtSH, mercaptoethanol; SRM, standard reaction mixture (0.13 ml).